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Award Number: DAMD17-00-1-0288

TITLE: Population Based Assessment of MHC Class I Antigens Down
Regulation as Markers of Increased Risk for Development
and Progression of Breast Cancer from Benign Breast
Lesions

PRINCIPAL INVESTIGATOR: Maria J. Worsham, Ph.D.

CONTRACTING ORGANIZATION: Henry Ford Health System
Detroit, Michigan 48202

REPORT DATE: June 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)**2. REPORT DATE**

June 2001

3. REPORT TYPE AND DATES COVERED

Annual (1 Jun 00 - 31 May 01)

4. TITLE AND SUBTITLE

Population Based Assessment of MHC Class I Antigens Down Regulation as Markers of Increased Risk for Development and Progression of Breast Cancer from Benign Breast Lesions

5. FUNDING NUMBERS

DAMD17-00-1-0288

6. AUTHOR(S)

Maria J. Worsham, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Henry Ford Health System
Detroit, Michigan 48202

E-Mail: mworsha1@hfhs.org**8. PERFORMING ORGANIZATION
REPORT NUMBER****9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING
AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES**

20011127 037

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 Words)**

Despite advances in chemotherapy and radiation therapies, advanced breast cancer still carries a high mortality rate. The need for effective therapies is urgent. The overall aim of this research proposal is to recognize early markers of disease and their interaction with other epidemiological risk factors that can serve as risk indicators for subsequent development of breast cancer from precancerous lesions, and as prognostic markers for progression from primary to metastatic disease. The major histocompatibility complex (MHC) class I molecules are found on the cell membrane of all cells in the body and are involved in intercellular communications and in complex interactions with the immune system. Cancer cells with reduced or aberrant MHC molecules have been shown to evade immune surveillance and become selected for cancer progression and spread of disease to distant sites of the body. About half of all breast cancers have complete loss or reduced level of MHC class I molecules and this finding has been associated with increased tumor invasiveness and more aggressive cancers with poorer outcome. The outlined studies are expected to better define the clinical significance of abnormal MHC class I molecules in precancerous and invasive breast lesions as markers of immunological events that could affect survival, selection, and outgrowth of precancerous cells, and their subsequent progression to breast cancer. These MHC losses could also mark more aggressive tumors and thus contribute to selection of appropriate treatments in individual cases.

14. SUBJECT TERMS

Major histocompatibility complex (MHC) class I molecules, disease progression markers, survival

15. NUMBER OF PAGES

33

16. PRICE CODE**17. SECURITY CLASSIFICATION
OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION
OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION
OF ABSTRACT**

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	6
Conclusions.....	6
References.....	7
Appendices.....	8

INTRODUCTION

It has been known for some time that malignant transformation of cells is frequently associated with abnormalities in the expression of MHC class I antigens (1). These abnormalities appear to play a role in the clinical course of the disease (1) and to have a negative effect on the outcome of T cell-based immunotherapy for malignant diseases (2, 3). In breast lesions examined for expression of MHC class I, approximately half (51%) of carcinomas had an abnormally low content of HLA-A, -B, and -C determinants (4). Down regulation of HLA class I antigens in breast carcinomas may be more frequent than previously reported suggesting that alterations of HLA class I could represent an important step associated with tumor invasion providing tumor cells with the ability to escape recognition by T-lymphocytes (5). The overall aim of our research is to better define the role of MHC class I antigen loss and its interaction with other histo-pathologic and epidemiological factors that can serve as risk indicators in the progression of primary breast cancer to metastatic disease.

BODY: Statement of Work

TASK 1:

In women with primary and metastatic lesions of the breast to determine whether HLA Class I antigen loss and down regulation is greater in those with late stage and metastatic disease than in women with early stage disease (months 1-48); to determine whether among women with concurrent preneoplastic lesions and breast tumors HLA Class I antigen loss or down regulation is more frequent in the tumor than in the pre-neoplastic lesion (1-54); association with histopathologic characteristics of the lesions, including estrogen and progesterone receptor status (months 1-54); and disease survival (1-58).

- a: Begin construction of the breast cancer cohort (3000 cases). The Pathologist Dr.Raju and the P.I will begin screening breast cancer cases for delineation into Stage1-IV, and for the presence of concurrent lesions of benign proliferative and cancer lesions, together with normal breast tissue. We will design appropriate forms to record histopathologic and clinical data based on our current NIH project forms and instruments (Instruments section in original grant)
- b: Retrieval of H & E slides for cases
- c: Review of slides
- d: selection of tumor blocks and sectioning of tissue for immunohistochemistry assays
- e: begin HLA class I immunoassays, as slides become available
- f: Continue construction of the breast cancer cohort, the concurrent lesion cohort and the histopathologic data gathering. See Pathology Review Form (PRF) (Instruments Section) for histopathologic

parameters.

- g: continue HLA class I immunoassays as additional cases are entered into the cohort
- h: Annual reports will be written
- i: Initial manuscripts on the PBD cohort will be written

PROGRESS (JANUARY 1, 2001-JUNE 30, 2001):

1. The Pathology Review Form (PRF) has been finalized (Appendix A)
2. Thus far 1,115 pathology reports have been obtained
3. H& E slides have been retrieved from the pathology archives for 250 cases
4. 200 cases have been reviewed by Dr. Raju on PRF forms
5. Selection of tumor blocks and sectioning of tissue for immunohistochemistry assays: completed for 170 cases
6. HLA class I immunoassays: completed for 60 cases
7. Medical abstraction form finalized (Appendix A)
8. Medical abstraction form: completed in 100 cases
9. Presented a paper (15th European Histocompatibility Conference) of a preliminary study of 12 cases (Appendix B).

TASK 2

Final analysis and report writing (months 56-60)

- a: Final analysis of epidemiological risk factor data, histopathological and clinical data and HLA expression results will be performed.
- b: A final report and additional manuscripts on the breast cancer cohort will be prepared

Progress: PENDING

KEY RESEARCH ACCOMPLISHMENTS

- Begun the compilation of a 3000 subject cohort of primary breast cancer patients
- Completed a multicenter validation of HLA immunoassays (Appendix B)
- Pilot study of 12 cases

REPORTABLE OUTCOMES

1. ABSTRACTS/PRESENTATIONS/MANUSCRIPTS

- a: HLA CLASS I AND II ANTIGEN EXPRESSION IN BREAST CARCINOMAS. R Nanavati, U Raju, S Ferrone, M J. Worsham. Department of Pathology, Henry Ford Health System, Detroit, Department of Immunology, Roswell Park Cancer Institute, Buffalo, 15th European Histocompatibility Conference, Granda Spain, March, 2001
- b: HLA ANTIGEN EXPRESSION IN BREAST CANCER: A MULTICENTRIC STUDY UTILIZING FORMALIN-FIXED PARAFFINIZED TISSUES. M J. Worsham¹, R. Nanavati¹, U. Raju¹, S.R. Wolman², T. Cabrera³, F. Garrido³, E. A. Repasky⁴, B. Hylander⁴, M. Feenstra⁵, M.Verdaasdonk⁵, M.Schipper⁵, M.Tilanus⁵, S.Ferrone⁴. ¹ Cancer Genetics Research, Department of Pathology, Henry Ford Health Systems, Detroit, MI, 48202, USA ² Uniformed Services Univ., of the Health Sciences, Bethesda, MD 20814, USA, Hosp., Univ., Virgen de las Nieves, Granada, Spain, ⁴ Roswell Park Cancer Institute, Buffalo, NY 14263, ⁵ Univ., Hosp., Utrecht, The Netherlands

CONCLUSIONS:

HLA CLASS I AND II ANTIGEN EXPRESSION IN BREAST CARCINOMAS

In the present study we have compared the expression of HLA antigens in breast carcinoma epithelial cells and in autologous normal epithelial cells utilizing 12 formalin-fixed, paraffin-embedded lesions. HLA class I antigen expression, measured by staining with mAb HC-10, was downregulated in 5 lesions, upregulated in 3, heterogeneous in 2, and similar to that in the autologous epithelial cells in 2. The results of the staining with mAb L368 were similar, but not superimposable, to those obtained with mAb HC-10. The 5 lesions which were stained by mAb L368 with a pattern similar to that of normal epithelial cells included 3 lesions with reduced staining by mAb HC-10 and 1 with an enhanced staining. The 3 lesions with an enhanced staining by mAb L368 displayed also an enhanced staining by mAb HC-10. The 4 lesions with a reduced staining by mAb L368 included 3 lesions with a reduced staining by mAb HC-10. A relationship was found between HLA class I antigen expression and the degree of differentiation of malignant cells. Neither breast carcinoma cells nor normal mammary epithelial cells were stained by anti-HLA class II mAb LGII-612.14. The only cells to be stained by mAb LGII-612.14 in the tissue sections analyzed were lymphocytes and dendritic cells, which were also identified by staining with an anti-CD45 mAb. These findings suggest that abnormalities in HLA class I antigen

expression are frequently associated with malignant transformation of mammary epithelial cells.

HLA ANTIGEN EXPRESSION IN BREAST CANCER: A MULTICENTRIC STUDY UTILIZING FORMALIN-FIXED PARAFFINIZED TISSUES.

Despite the possible clinical significance and potential for T-cell based immunotherapy, evaluation of malignant lesions for HLA class I antigen expression is not performed routinely, even for patients who are candidates for such therapy. This reflects, at least in part, reluctance by pathologists to utilize frozen tissue sections in IHC assays. Little information is available about the usefulness of formalin-fixed paraffin-embedded tissues (FFPT) as substrates in IHC assays to evaluate tissue expression of HLA antigens. We therefore undertook a multicentric study to develop and standardize an IHC protocol using FFPTs and anti-HLA mAbs. To determine if loss of expression of MHC Class I molecules at the protein level reflect alterations at the gene level, DNA from microdissected normal and tumor tissue were evaluated with microsatellites at the MHC class I 6p21.3 locus (HLA-A, B, C determinants) and at the 15q21 beta 2 microglobulin locus for concordance of expression. HLA class I antigen down-regulation in conjunction with cellular heterogeneity of expression in three breast carcinoma cases was concordantly reported by the four participating laboratories with the anti-HLA class I antibody HC-10 and with the anti-beta 2 microglobulin L368. Furthermore, no staining of normal and malignant mammary cells was detected by the four laboratories in the lesions stained with the anti-HLA class II LGII. In contrast, infiltrating lymphocytes were strongly stained by LGII. Downregulation of class was reflected by LOH in cases 1 and 3 for the 15q21 locus and in case 1 at the 6p21 locus. The results indicate that FFPTs represent a useful substrate upon which to monitor HLA antigen expression in malignant lesions, especially when appropriate markers are used to differentiate malignant cells from lymphocytes and dendritic cells.

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3. Jager E, Ringhoffer M, Karbach J, Arand M, Oesch F, Jager E, Knuth A. Immunoselection in vivo: independent loss of MHC class I and melanocyte differentiation antigen expression in metastatic melanoma mediated by antigen-specific CTL. *Int. J. Cancer* 71:142-147, 1997
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5. Maiorana AM, Cesinaro AM, Fano RA, Collina G. Expression of MHC class I and class II antigens in primary breast carcinomas and synchronous nodal metastases. *Clin Exp Metastasis* 13: 43-48, 1995

APPENDICES

A: Study Instruments

- a: Pathology Review Form
- b: Medical Record Abstraction Form

B: Presented Abstracts:

- a: HLA class I and II antigen expression in breast carcinomas
- b: HLA antigen expression in breast cancer: a multicentric study utilizing formalin-fixed paraffinized tissues

CANCER PATHOLOGY REVIEW FORM

PLACE LABEL HERE:

MRN

Pathology # Specimen #

Date of Pathology Report

BIOPSY REVIEWER

☐ No ☐ Yes Usha Raju
☐ No ☐ Yes Maria J. Worsham

FORM COMPLETION DATE

___/___/___

TYPE OF TISSUE SAMPLE

- ☐ Needle biopsy
☐ Excision biopsy
☐ Simple Mastectomy
☐ Modified Radical Mastectomy
Uncertain
☐ Other _____
☐ Unknown

LOCALIZATION

- ☐ No
☐ Yes
☐ Unknown

SOURCE OF BREAST TISSUE

- ☐ Left
☐ Right
☐ Both
☐ Unknown

BREAST QUADRANT

- ☐ Upper Inner ☐ Upper Outer
☐ Lower Inner ☐ Lower Outer
☐ Central ☐ Unknown

GROSS FINDINGS

- ☐ No lesion
☐ Cyst(s) ⇒ ☐ Solitary ☐ Multiple
☐ Mass(es) ⇒ ☐ Solitary ☐ Multiple

Size of Largest Mass/Cyst ____ . ____ cm

- ☐ Other _____ ☐ Unknown

MICROSCOPIC FINDINGS

MALIGNANCY

Present ☐ No ☐ Yes

FOCI

SIZE

BLOCK

TYPE:

☐

☐ Invasive ductal

☐ >1 specify number ____

☐ Invasive lobular

☐ > multifocal

☐ Tubular

☐ Mucinous

☐ Medullary

☐ Other

Angiolymphatic Invasion ☐ No ☐ Yes
Prominent Lymphocytic infiltrate ☐ No ☐ Yes

Necrosis ☐ No ☐ Yes

DCIS COMPONENT

☐ EIC+ ____ %

☐ EIC-

Margins ☐ No ☐ Yes

Lymphnodes: ☐ Negative ☐ Positive

BORDER:

Pushing ☐ No ☐ Yes

Infiltrative ☐ No ☐ Yes

GRADE:

Nuclear 1 2 3

Tubules 1 2 3

Mitotic 1 2 3

Number Sampled ____ Number Positive ____

ASSOCIATED LESIONS

FIBROADENOMA ☐ Simple ☐ Complex

SIMPLE ADENOSIS (Mod-florid)

SCLEROSING ADENOSIS (Mod-florid)

APOCRINE ADENOSIS (Mod-florid)

HYPERPLASIA WITHOUT ATYPIA (USUAL TYPE) (Mod-florid)

HYPERPLASIA WITHOUT ATYPIA (APOCRINE TYPE) (Mod-florid)

ADH*

ALH*

PAPILLOMA

RADIAL SCAR

LCIS*

DCIS*

☐ No ☐ Yes

☐ No ☐ Yes

☐ No ☐ Yes

☐ No ☐ Yes

☐ No ☐ Yes

☐ No ☐ Yes

☐ No ☐ Yes

☐ No ☐ Yes

☐ No ☐ Yes

☐ No ☐ Yes

☐ No ☐ Yes

☐ No ☐ Yes

*ADH: Atypical Ductal Hyperplasia

*ALH: Atypical Lobular Hyperplasia

*LCIS: Lobular Carcinoma In Situ

*DCIS: Ductal Carcinoma In Situ

BENIGN BREAST DISEASE STUDY

MEDICAL RECORD ABSTRACT

MRN _____	Follow-up Complete	Yes	No
Index Date ____ / ____ / ____	Abstractor Status:	1. Complete/Finalized 2. Incomplete/Finalized 3. Chart Not Received	
Date Abstracted ____ / ____ / ____	Abstractor's Initials _____		

DEMOGRAPHICS at time of chart abstraction:

1. Name:

_____ [last] _____ [first] _____ [mi]

2. Social Security Number:

_____ - ____ - _____

3. Date of Birth:

____ / ____ / _____

4. Sex:

1=Female
2=Male

5. Race:

1=White/Caucasian
2=Black/African American
3=Hispanic/Latino
4=Asian/Pacific Islander
5=Middle Eastern
6=Native American/American Indian
7=Other, specify _____
9=Unknown

6. Current Marital Status:

1=Divorced
2=Married
3=Single
4=Widowed
5=Legally separated
9=Unknown

7. Spouse's Name, if applicable: _____

8. Maiden Name: _____

9. Former Last Name: _____

10. Vital Status: 0=Deceased
 1=Alive

11. Date of Vital Status Assessment: ____ / ____ / ____

12. Insurance at Index Date: 1=HAP Date documented: ____ / ____ / ____
 2=Other HMO
 3=Blue Cross/Blue Shield
 4=Medicare
 5=Medicaid
 6=Other _____
 7=None
 9=Unknown

13. Previous Insurance: 1=HAP Date documented: ____ / ____ / ____
 (w/in 10 yrs prior to index) 2=Other HMO
 3=Blue Cross/Blue Shield
 4=Medicare
 5=Medicaid
 6=Other _____
 7=None
 9=Unknown

14. Highest Education: 1=Grade School (< 8 years)
 2=Some High School (8 – 11 years)
 3=Completed High School/GED
 4=Vocational School
 5=Some College
 6=Completed College
 7=Post-graduate School
 9=Unknown

3. Other Medical Conditions diagnosed/mentioned
up to 10 years prior to index date:

0=No
1=Yes
9=Unknown

Allergies:

____ Drug allergy
____ Food allergy
____ Hay fever
____ Other allergies

____ Anemia or other blood disorder
____ Arthritis (Non-inflammatory)
____ Arthritis (Rheumatoid)

Cardiovascular Diseases:

____ Heart disease
____ Hypertension (high blood pressure)

Cerebrovascular Diseases:

____ Stroke
____ Transient Ischemic Attack (TIA)

____ Diabetes ('sugar')
____ Folate deficiency
____ Hyperthyroid disease
____ Hypoglycemia
____ Hypothyroid disease
____ Immune system disorder

Infectious Diseases:

____ Chicken pox
____ Encephalitis
____ Herpes simplex
____ Measles
____ Meningitis
____ Mononucleosis (mono)
____ Mumps
____ Pneumonia

Infectious Diseases (cont.):

____ Poliomyelitis (polio)
____ Shingles zoster
____ Toxoplasmosis
____ Tuberculosis (TB)
____ Typhoid

____ Kidney disease
____ Liver disease

Neurologic/Psychiatric Disorders:

____ Clinical depression
____ Epilepsy/Seizures/Convulsions
____ Migraine headaches
____ Multiple Sclerosis (MS)
____ Psychiatric conditions requiring medication

____ Parathyroid disease
____ Pituitary disease

Respiratory Diseases:

____ Asthma
____ Emphysema
____ Other respiratory disease

____ Stomach or other digestive disorder
____ Vitamin B1 Deficiency
____ Vitamin B12 Deficiency

Other Medical Conditions (specify):

4. Mammography History from beginning up to one year after index date: 0=No
1=Yes
9=Unknown

<u>Dates:</u>	<u>Results:</u>	<u>Result Codes:</u>
____/____/____	_____	1=Negative
____/____/____	_____	2=Benign/Negative
____/____/____	_____	3=Probably Benign
____/____/____	_____	4=Suspicious
____/____/____	_____	5=Highly Suspicious
____/____/____	_____	8=Incomplete/Inconclusive
____/____/____	_____	9=Unknown

5. Breast Biopsy History from beginning up to one year after index date: 0=No
1=Yes
9=Unknown

<u>Dates:</u>	<u>Results:</u>	<u>Result Codes:</u>
____/____/____	_____	1= Benign Breast Disease (BBD)
____/____/____	_____	2= Ductal or Lobular Carcinoma In Situ (DCIS or LCIS)
____/____/____	_____	3= Cancer (Carcinoma)
____/____/____	_____	4= Both BBD and CIS/Cancer
____/____/____	_____	5= Lumpectomy or Mastectomy (unilateral or bilateral)
____/____/____	_____	6= Cosmetic Breast Reduction or Enlargement
____/____/____	_____	7= Other Breast Biopsy (non-mammary epithelial biopsy of skin, nipple, fat, axillary lymph nodes, etc.)
____/____/____	_____	8= Incomplete/Inconclusive
____/____/____	_____	9=Unknown

BODY SIZE INFORMATION

1. Maximum Height (inches): _____ Date: ____ / ____ / ____
2. Weight closest to **index date** (pounds): _____ Date: ____ / ____ / ____
3. Weight during previous decade (pounds): _____ Date: ____ / ____ / ____
4. Weight during previous decade (pounds): _____ Date: ____ / ____ / ____
5. Weight during previous decade (pounds): _____ Date: ____ / ____ / ____

REPRODUCTIVE HISTORY from beginning of chart up to index date:

1. Age at Menarche (years): _____ [99=Unknown]
 2. Age at First Birth (years): _____ [88=No Children; 99=Unknown]
 3. Number of Pregnancies up to **index date**: _____ [99=Unknown]
 4. Total Number of Pregnancies (gravida): _____ [99=Unknown]
 5. Total Number of Births (para): _____ [99=Unknown]
 6. Menopausal Status at **index date**:
 1=Pre-menopausal
 2=Peri-menopausal
 3=Post-menopausal
 9=Unknown
 7. Year of Menopause:
 (skip if not post-menopausal) _____
 8. Hysterectomy:
 0=No
 1=Yes
 9=Unknown
- Number of ovaries removed: _____
- Date of surgery: ____ / ____ / ____

CANCER HISTORY

1. Subject's History of Primary Cancer from beginning of chart up to index date: 0=No
1=Yes
9=Unknown

	<u>Yes/No:</u>	<u>Date of Dx:</u>
A. Breast	_____	____ / ____ / ____
B. Endometrial	_____	____ / ____ / ____
C. Colorectal	_____	____ / ____ / ____
D. Ovarian	_____	____ / ____ / ____
E. Cervical	_____	____ / ____ / ____
F. Other:		
site: _____ code: _____	_____	____ / ____ / ____
site: _____ code: _____	_____	____ / ____ / ____

2. Family History of Cancer from beginning of chart up to index date: 0=No
1=Yes
9=Unknown

	<u>Relative:</u>	<u>Rel. Code:</u>	<u>Cancer:</u>	<u>Cancer Code:</u>	<u>Age at Dx:</u>
A.	_____	_____	_____	_____	_____
B.	_____	_____	_____	_____	_____
C.	_____	_____	_____	_____	_____
D.	_____	_____	_____	_____	_____
E.	_____	_____	_____	_____	_____
F.	_____	_____	_____	_____	_____
G.	_____	_____	_____	_____	_____
H.	_____	_____	_____	_____	_____

LIFESTYLE HISTORY

1. Smoking Status starting with beginning of chart up to index date:

0=No

1=Yes

9=Unknown

Date	Status: 1=Current Smoker 2=Past Smoker 3=Never Smoker	Packs/Day*	# of Years at this Packs/Day	If Past Smoker, Calendar Year Quit
____/____/____				
____/____/____				
____/____/____				

<u>*Cigarettes/day</u>	<u>Packs/day</u>
1 - 5	0.25
6 - 10	0.50
11 - 15	0.75
16 - 20	1.0

2. Occupational History within 10 years of index date:

0=No

1=Yes

9=Unknown

Date	Name of Occupation	Years in Occupation
____/____/____		
____/____/____		
____/____/____		

IF INCOMPLETE FOLLOW-UP:

1. Telephone numbers: Home: (____) _____ Work: (____) _____

Emergency: (____) _____

2. Current address: Street Address _____

City, State, Zip Code _____

3. Address at index date, if different: _____ Date: ____ / ____ / ____

4. Previous address, if different: _____ Date: ____ / ____ / ____

5. Spouse's employer: Name _____ Phone #: (____) _____

City, State _____

6. Name and address of next-of-kin: _____

Relationship:

1. Current spouse
2. Former spouse
3. Offspring
4. Parent
5. Sibling
6. Other, specify _____

7. Date of last physician visit or hospital admission: ____ / ____ / ____

8. Name of primary care physician: _____

9. Location of primary care physician: _____

HLA CLASS I AND II ANTIGEN EXPRESSION IN BREAST CARCINOMAS.

R Nanavati, U Raju, S Ferrone, M J. Worsham. Department of Pathology, Henry Ford Health System, Detroit, Department of Immunology, Roswell Park Cancer Institute, Buffalo

Abstract

Immunohistochemical staining of a large number of frozen sections of surgically removed lesions has shown that malignant transformation of cells may be associated with changes in their HLA phenotype. Since the use of frozen tissue sections as a substrate in immunohistochemical reactions has hindered the routine analysis of HLA antigen expression in malignant lesions, we have been evaluating the expression of HLA antigens in formalin-fixed, paraffin-embedded lesions. The anti-HLA class I heavy chain mAb HC-10, the anti- β_2m mAb L368 and the anti-HLA class II mAb LGII-612.14 have been used as probes. In the present study we have compared the expression of HLA antigens in breast carcinoma epithelial cells and in autologous normal epithelial cells utilizing 12 formalin-fixed, paraffin-embedded lesions. HLA class I antigen expression, measured by staining with mAb HC-10, was downregulated in 5 lesions, upregulated in 3, heterogeneous in 2, and similar to that in the autologous epithelial cells in 2. The results of the staining with mAb L368 were similar, but not superimposable, to those obtained with mAb HC-10. The 5 lesions which were stained by mAb L368 with a pattern similar to that of normal epithelial cells included 3 lesions with reduced staining by mAb HC-10 and 1 with an enhanced staining. The 3 lesions with an enhanced staining by mAb L368 displayed also an enhanced staining by mAb HC-10. The 4 lesions with a reduced staining by mAb L368 included 3 lesions with a reduced staining by mAb HC-10. A relationship was found between HLA class I antigen expression and the degree of differentiation of malignant cells. Neither breast carcinoma cells nor normal mammary epithelial cells were stained by anti-HLA class II mAb LGII-612.14. The only cells to be stained by mAb LGII-612.14 in the tissue sections analyzed were lymphocytes and dendritic cells, which were also identified by staining with an anti-CD45 mAb. These findings suggest that abnormalities in HLA class I antigen expression are frequently associated with malignant transformation of mammary epithelial cells.

Supported by DOD DAMD 17-00-1-0288 and the Fund for Henry Ford Hospital

BACKGROUND

Complete loss as well as allele- and locus-specific downregulation of the Major Histocompatibility Complex (MHC) class I expression occurs frequently in human tumors of different origins. Tumor cells can only be recognized by cytotoxic T cells if they express the same tumor antigen in conjunction with a MHC class I molecule. Reduced MHC class I expression may present an "escape route" for neoplastic cells constituting a selective advantage for survival of precancerous cells, and permitting their subsequent outgrowth or metastasis. This lack of recognition of the tumor cell could result in diminished returns for T cell-based immunotherapy for malignant diseases.

Approximately half (51%) of human breast cancers have an abnormally low content of HLA-A, B and C determinants (MHC class I). Abnormalities of MHC class I appear to be related to breast tumor invasiveness and with clinical and pathological parameters such as stage and degree of differentiation.

Over the past two decades the availability of HLA-specific monoclonal antibodies (mAb) suitable for immunohistochemical staining and technical advances in

immunohistochemical staining techniques have allowed extensive analysis of MHC class I antigen expression in cryopreserved tumors. These studies have shown conclusively that MHC class I antigen down regulation occurs in a number of malignant lesions, although with marked differences in frequency (for review, see 1).

Testing of surgically removed malignant lesions for HLA Class I antigen expression has not become standard-of-care for evaluation of patients with malignancies, even those to be enrolled in trials of T cell-based immunotherapy. This attitude reflects, at least in part, pathologists' reluctance to utilize frozen tissue sections in immunohistochemical assays, since very few anti-HLA class I mAb have been reported to stain formalin-fixed tissues.

We evaluated 12 breast carcinomas to assess HLA class I and II antigen abnormalities utilizing immunohistochemical staining in formalin-fixed paraffin tissues.

MATERIAL AND METHODS

Antibodies

In this study we evaluated three HLA antibodies that bind to respective epitopes in a background of formalin fixation (Table 1). Two HLA class I antibodies, HC10 and LG368, and one class II antibody, LGII, were evaluated in this study. Anti-HLA Class I mAbs, HC-10 (2, 3) and L368 (4) stain formalin-fixed tissue sections. mAb, HC-10 recognizes distinct determinants expressed on the heavy chains of many, but not all HLA Class I alleles. mAb L368 recognizes one determinant of beta2 microglobulin.

Table 1 **HLA Antibodies**

HLA Class I	HLA Class II	Other
HC10	LGII	CD45
L368		

Tumor Specimens

Formalin fixed paraffin tissue sections (5 microns in thickness) from 12 breast cancer cases with normal breast epithelium and cancer in the same tissue section were evaluated for HLA class I and class II expression.

Immunohistochemistry (IHC) Methodology

An IHC methodology, validated for HLA antigen assays in formalin fixed tissues in four laboratories was adopted. In brief, dilutions were as follows : mAb HC-10: 1/100 dilution, mAb L368: 1/25, mAb LGII612.14: 1/25, with overnight incubation for all three. The standardized manual methodology utilizes antigen-retrieval with endogenous peroxidase inactivation using 3% hydrogen peroxide, with normal horse serum as the blocking agent (Vector Laboratories, Burlingame, CA). Following incubation with monoclonal dilutions , the slides are washed and incubated with biotinylated horse anti-mouse immunoglobulin G (Vector Laboratories, Inc.), followed by incubation with avidin-biotin peroxidase complex (Vectastain Elite ABC Kit: Vector Laboratories, Inc.). Immunoreactivity is visualized with 3',3-diaminobenzidine tetrahydrochloride (Vector laboratories, Inc.) and the sections are counterstained with Mayer's Hematoxylin.

At the present time we have converted to an automated immunoperoxidase staining system (Ventana Medical Systems, Inc, Tucson, Az). Antigen retrieval is accomplished by steaming in an Black and Decker steamer, followed by titration using the same dilutions as the manual method and counterstained with DAB.

IHC Interpretation

Scoring of stain intensity in the normal/benign areas and the tumor are done with reference to staining intensity of normal breast epithelium present in the same section with the breast tumor. Stromal lymphocytes in lymphocytic infiltrations which stain intensely can also serve as a reference for normal "staining intensity".

Scoring parameters include cell membrane localization. Staining interpretation is derived as follows: 0-25% of cells = negative; <25%-50% = heterogeneous; >50%-75% = heterogeneous; (>25%-<75% = heterogeneous); >75% = positive staining. Scoring is done by two independent reviewers. Complete absence of staining as compared to the presence of staining in the normal breast epithelium is scored as "negative". Marked decrease in staining intensity is scored as "+", presence of some

staining intensity receives a score of "++", moderate stain intensity a score of "+++", and a score of "++++" indicates intensity of stain seen in normal breast tissue or stromal or lymphocytes in infiltrates.

RESULTS

HLA Expression

Table 2 ANTIBODY: HC10

Tissue area	NORMAL				TUMOR				Outcome
Staining score		Heterogenous				Heterogenous			
% cells stained	< 25%	> 25% < 50%	> 50% < 75%	> 75%	< 25%	> 25% < 50%	> 50% < 75%	> 75%	
Case 1			•		•a			•b	upregulation/ downregulation
Case 2	•						•		upregulation
Case 3				•	•	•			downregulation
Case 4	•		•					•	upregulation
Case 5		•						•	upregulation
Case 6	•				•				no change
Case 7		•			•a			•b	upregulation/ downregulation
Case 8			•		•				downregulation
case 9	•				•				no change
Case 10				•	•				downregulation
Case 11				•		•			downregulation
Case 12	•	•			•				downregulation

a: poorly differentiated areas of the tumor

b: well differentiated areas of the tumor

HLA Expression Results

Table 3 ANTIBODYL368

Tissue area	NORMAL				TUMOR				Outcome
Staining score		Heterogenous				Heterogenous			
% cells stained	< 25%	> 25% < 50%	> 50% < 75%	> 75%	< 25%	> 25% < 50%	> 50% < 75%	> 75%	
Case 1	•					•			upregulation
Case 2	•						•		upregulation
Case 3		•				•			no change
Case 4			•					•	upregulation
Case 5	•				•				no change
Case 6	•				•				no change
Case 7		•			•a		• b		downregulation: heterogeneous
Case 8	•	•			•				downregulation
case 9		•			•				downregulation
Case 10	•				•				no change
Case 11		•				•			no change
Case 12		•			•a		•b		downregulation: heterogeneous

a: poorly differentiated areas of the tumor

b: well differentiated areas of the tumor

Table 4 Summary of HLA class I Antibodies

	Outcome HC10	Outcome L368
Case 1	upregulation/downregulation	upregulation
Case2	upregulation	upregulation
Case 3	downregulation	no change
Case 4	upregulation	upregulation
Case 5	upregulation	no change
Case 6	no change	no change
Case 7	upregulation/downregulation	downregulation:heterogeneous
Case 8	downregulation	downregulation
case 9	no change	downregulation
Case 10	downregulation	no change
Case 11	downregulation	no change
Case 12	downregulation	downregulation:heterogeneous

SUMMARY

- Aberrant class I expression in breast tumors appears to be a more frequent phenomenon than previously reported.
- Upregulation of L368 appears to occur in conjunction with upregulation of HC10
- Downregulation of HC10 (6p21 loci) may be a more frequent event occurring together with and independent of L368 (15q21 loci).

DISCUSSION

The major histocompatibility complex (MHC) class I molecules are found on the cell membrane of all cells in the body and are involved in intercellular communications and in complex interactions with the immune system. Cancer cells with reduced or aberrant MHC molecules have been shown to evade immune surveillance and become selected for cancer progression and spread of disease to distant sites of the body.

About half of all breast cancers have complete loss or reduced level of MHC class I molecules and this finding has been associated with increased tumor invasiveness and more aggressive cancers with poorer outcome.

The outlined studies are expected to better define the clinical significance of

abnormal MHC class I molecules in invasive breast lesions as markers of immunological events that target more aggressive tumors and thus contribute to selection of appropriate treatments in individual cases.

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ANTIGEN EXPRESSION IN BREAST CANCER: A MULTICENTRIC STUDY UTILIZING FORMALIN FIXED TISSUES.

Maria J. Worsham, Reshma Nanavati, Usha Raju, Sandra R. Wolman, Teresa Cabrera, Federico Garrido, Elizabeth Reparsky, Bonnie Hylander, Manita Feenstra, Marina Verdaasdonk, Marguerite Schipper, Marcel Tilanus, Soldano Ferrone. Cancer Genetics Research, Department of Pathology, Henry Ford Health Systems, Detroit, MI; Hospital Universitario Virgen de las Nieves, Granada, Spain; Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY; University Hospital, Utrecht, The Netherlands; Uniformed Services University of the Health Sciences, Bethesda, MD

ABSTRACT

Immunohistochemical (IHC) staining of frozen malignant lesions has shown that abnormalities in HLA class I antigen expression are frequent in malignant cells. Although these abnormalities appear to have clinical significance, evaluation of malignant lesions for HLA class I antigen expression is not a routine procedure, even for those patients to be treated with T cell-based immunotherapy. This reflects, at least in part, pathologists' reluctance to utilize frozen tissue sections in IHC assays.

Scanty information is available about the usefulness of formalin fixed tissues as a substrate in IHC assays to evaluate HLA antigen expression in tissues. We therefore undertook a multicentric study to develop and standardize an IHC protocol using formalin fixed tissues and anti-HLA mAbs.

HLA class I antigen downregulation in two of three breast carcinoma lesions in conjunction with heterogeneity was concordantly scored by the four participating laboratories with the anti-HLA class I mAb HC-10 and with the anti-beta 2 microglobulin mAb L368. Furthermore, no staining of normal and malignant mammary cells was detected by the four laboratories in the lesions stained with the anti-HLA class II mAb LGII-612.14. In contrast, infiltrating lymphocytes were strongly stained by mAb LGII. These results indicate that formalin fixed tissue sections represent a useful substrate to monitor HLA antigen expression in malignant lesions, especially when appropriate markers are also used to differentiate malignant cells from lymphocytes and dendritic cells.

Supported by DAMD17-00-1-0288

BACKGROUND

Tumor immunology has been a subject of great interest for over a century. Improved molecular understanding of human tumor immunity and recent technical advances have made immunotherapy (e.g., peptide vaccination, adoptive immunotherapy) a promising modality of treatment.

Over the past two decades the availability of HLA-specific monoclonal antibodies (mAb) suitable for immunohistochemical staining and technical advances in immunohistochemical staining techniques have allowed extensive analysis of MHC class I antigen expression in cryopreserved tumors. These studies have shown conclusively that MHC class I antigen down regulation occurs in a number of malignant lesions, although

with marked differences in frequency (for review, see 1). The frequency of HLA Class I antigen down regulation is significantly higher ($p < 0.01$) in breast carcinoma (51%) and prostate carcinoma (85%) than it is in head and neck squamous cell carcinoma, lung carcinoma, colon carcinoma, cervical carcinoma or melanoma (1). In human breast cancers, MHC class I antigen expression appears to be related inversely to tumor invasiveness (2) and is strongly associated with clinico-pathological parameters such as lower tumor stage and degree of differentiation (3, 4). Furthermore, in a limited number of patients, MHC class I antigen down regulation in metastatic lesions appears to have a negative impact on the outcome of T cell-based immunotherapy of malignant diseases. Reduction or loss of HLA Class I antigen expression in metastatic lesions is associated with disease progression (5) or with recurrence of the disease in patients treated with T cell-based immunotherapy (6, 7). In spite of these results, testing of surgically removed malignant lesions for HLA Class I antigen expression has not become standard-of-care for evaluation of patients with malignancies, even those to be enrolled in trials of T cell-based immunotherapy. This attitude reflects, at least in part, pathologists' reluctance to utilize frozen tissue sections in immunohistochemical assays, since very few anti-HLA class I mAb have been reported to stain formalin-fixed tissues.

We therefore undertook a multicenter study to standardize methodology and reagents in order to assess the validity of immunohistochemical staining in formalin-fixed paraffin tissues for HLA class I antigen abnormalities.

MATERIAL AND METHODS

Antibodies

In this study we evaluated three HLA antibodies that bind to respective epitopes in a background of formalin fixation (Table 1). Two HLA class I antibodies, HC-10 and LG-368, and one class II antibody, LGII, were evaluated in this study. The same batch of antibodies was provided by Laboratory 1 (Ferrone) to the three other laboratories: Laboratory 2-(Garrido), Laboratory 3 (Tilanus), and Laboratory 4 (Worsham). Anti-HLA Class I mAbs, HC-10 (14, 15) and L368 (16) stain formalin-fixed tissue sections. mAb, HC-10 recognizes distinct determinants expressed on the heavy chains of many, but not all HLA Class I alleles and may underestimate the expression of HLA Class I alleles in malignant lesions or even yield false negative results. mAb L368 recognizes one determinant of the beta2- microglobulin at the 15q21 locus.

Anti-CD45 staining was also done to confirm the presence of lymphocytes and dendritic cells in tumor infiltrates.

Tumor Specimens

Formalin fixed paraffin tissue sections (5 microns in thickness) from three breast cancer cases that spanned different years (to test the consistency of the methodology where formalin fixation might be a variable) were provided by Laboratory 4 to the other

participants. In all cases, normal or benign breast epithelium was present in addition to tumor on the sections.

Immunohistochemistry (IHC) Methodology

A validated IHC methodology was employed. In brief, dilutions were as follows : mAb HC-10: 1/100 dilution, mAb L368: 1/25, mAb LGII612.14: 1/25, with overnight incubation for all three. The standardized methodology utilizes antigen-retrieval with endogenous peroxidase inactivation using 3% hydrogen peroxide, with normal horse serum as the blocking agent (Vector Laboratories, Burlingame, CA). Following incubation with monoclonal dilutions , the slides are washed and incubated with biotinylated horse anti-mouse immunoglobulin G (Vector Laboratories, Inc.), followed by incubation with avidin-biotin peroxidase complex (Vectastain Elite ABC Kit: Vector Laboratories, Inc.). Immunoreactivity is visualized with 3',3-diaminobenzidine tetrahydrochloride (Vector laboratories, Inc.) and the sections are counterstained with Mayer's Hematoxylin.

IHC Interpretation

Scoring of stain intensity in the benign lesion areas and the tumor are done with reference to staining intensity of normal breast epithelium present either in the same section with the breast tumor, or processed concurrently with the tumor section. Stromal lymphocytes in lymphocytic infiltrations which stain intensely can also serve as a reference for normal "staining intensity".

Scoring parameters include cell membrane localization. Staining interpretation is derived as follows: 0-25% of cells = negative; <25%-50% = heterogeneous; >50%-75% = heterogeneous; (>25%-<75% = heterogeneous); >75% = positive staining. Scoring is done by two independent reviewers in each of the four centers in a blinded fashion. Complete absence of staining as compared to the presence of staining in the normal breast epithelium is scored as "negative". Marked decrease in staining intensity is scored as "+", presence of some staining intensity receives a score of "++", moderate stain intensity a score of "+++", and a score of "++++" indicates intensity of stain seen in normal breast tissue or lymphocytes in infiltrates.

RESULTS

DISCUSSION

The potential predictive value of HLA expression in evaluation and therapy planning has been under-utilized. The importance of this marker in evaluation of breast cancer patients prompted our undertaking of a multicentric study approach to develop and standardize an IHC protocol using formalin-fixed, paraffin-embedded tissues and anti-HLA mAbs. HC-10, a mAb to a determinant expressed on free HLA class I heavy chains (chromosome 6p21) and L368, an anti-human beta2- microglobulin (chromosome 15q21)

were evaluated in archival formalin-fixed paraffin embedded tissue section.

HLA class I antigen downregulation in conjunction with and without heterogeneity was concordantly scored in tumor regions of all cases with HC10 and mAb L368. Furthermore, no staining of either normal and malignant mammary cells was detected by the four laboratories in lesions stained with LGII, although infiltrating lymphocytes were strongly stained. While there was some lack of agreement with staining interpretations in normal breast epithelium for L368, lymphocytes and stromal cells were judged positively stained by all four laboratories.

The results indicate that formalin fixed paraffin tissues represent a useful substrate to monitor HLA antigen expression in malignant lesions, especially when appropriate markers are also used to differentiate malignant cells from lymphocytes and dendritic cells.

Lack of staining of a lesion with one mAb does not always reflect loss of the molecule expressing the tested determinant. Assessment of the HLA expression with either HC-10 or L368 may yield false negative results. To overcome the limitations posed by false negatives, mAbs to distinct monomorphic determinants on the heavy chains of all HLA Class I alleles should provide additional evidence for HLA expression in formalin fixed tissues.

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Table 1 HLA Antibodies

HLA Class I	HLA Class II	Other
HC-10	LGII	CD45
L368		

Table 2 Summary of Validation Results

Results	Case 1						Case 2						Case 3					
	HC-10		L368		LGII		HC-10		L368		LGII		HC-10		L368		LGII	
	Normal	Tumor	Normal	Tumor	Normal	Tumor	Normal	Tumor	Normal	Tumor	Normal	Tumor	Normal	Tumor	Normal	Tumor	Normal	Tumor
Antibody																		
Lab 1	†	†	†	†	†	†	†	†	†*	†	†*	†	†	†	†*	†	†	†
Lab 2	†	†	†	†	†	†	†	†	†	†	†	†	†	†	†**	†	†	†
Lab 3	†	†	†*	†	†	†	†	†	†	†	†	†	†	†	†**	†	†	†
Lab 4	†	†	†	†	†	†	†*	†	†	†	†	†	†	†	†**	†	†	†

† = complete concordance

† = incomplete concordance

†* = complete concordance, see text

†* = incomplete concordance, see text

†** = stromal cells and lymphocytes

Figure legends HLA

Figure 1: Normal breast epithelium from case 1 illustrating uniform membrane “+++” stain intensity with HC-10

Figure 2: Concurrent invasion lesion from Case 1 showing reduced expression of HC-10. Note stromal cells and scattered lymphocytes with “+++” staining intensity.